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STUDIES ON THE SUCCINATE DEHYDROGENATING SYSTEM

I. KINETICS OF THE SUCCINATE DEHYDROGENASE INTERACTION WITH A SEMIQUINDIIMINE RADICAL OF *N,N,N',N'*-TETRAMETHYL-*p*-PHENYLENEDIAMINE

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Summary

1. The activities of the soluble reconstitutively active succinate dehydrogenase (EC 1.3.99.1) measured with three artificial electron acceptors, e.g. ferricyanide, phenazine methosulfate and free radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (WB), have been compared. The values estimated by extrapolation to infinite acceptor concentration using double reciprocal plots $1/v$ versus 1/[acceptor] are nearly the same for ferricyanide and phenazine methosulfate and about twice as high for the WB.

2. The double reciprocal plots $1/v$ versus 1/[succinate] in the presence of malonate at various concentrations of WB give a series of straight lines intercepting in the third quadrant. The data support the mechanism of the overall reaction, in which the reduced enzyme is oxidized by WB before dissociation of the enzyme-product complex.

3. The dependence of the rate of the overall reaction on WB concentration shows that only one kinetically significant redox site of the soluble succinate dehydrogenase is involved in the reduction of WB.

4. Studies of the change of V and K_m values during aerobic inactivation of the soluble enzyme suggest that only 'the low K_m ferricyanide reactive site' (Vinogradov, A.D., Gavrikova, E.V. and Goloveshkina, V.G. (1975) Biochem. Biophys. Res. Commun. 65, 1264—1269) is involved in reoxidation of the reduced enzyme by WB.

5. The pH dependence of V for the succinate-WB reductase reaction shows

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; WB, Wurster's Blue, a semiquindiimine radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; HiPIP denotes the high-potential Fe-S cluster.

that the group of the enzyme with the pK_a value of 6.7 at 22°C is responsible for the reduction of dehydrogenase in the enzyme-substrate complex.

6. When WB interacts with the succinate-ubiquinone region of the respiratory chain, the double reciprocal plot $1/v$ versus $1/[WB]$ gives a straight line. The thenoyltrifluoroacetone inhibition of succinate-ubiquinone reductase or extraction of ubiquinone alter the $1/v$ versus $1/[WB]$ plots for the curves with a positive initial slope intercepting the ordinate at the same V as in the native particles. The data support the mechanism of succinate-ubiquinone reduction, in which no positive modulation of succinate dehydrogenase by ubiquinone exist in the membrane.

Introduction

Being a part of the multienzyme complex of the respiratory chain, succinate dehydrogenase (EC 1.3.99.1) catalyzes the reversible two-substrate reaction:

$$\text{Succinate}^{2-} + A_{ox} \leftrightarrow \text{Fumarate}^{2-} + A_{red} \quad (1)$$

where A_{ox} and A_{red} are oxidized and reduced species of the electron acceptor. When the membrane-bound succinate dehydrogenating system is operating, ubiquinone serves as an immediate electron acceptor in reaction 1 [1—3]. The purified soluble preparations of succinate dehydrogenase do not directly react with coenzyme Q [4—6]. Two approaches are widely employed for the measurement of catalytic activity of the soluble enzyme: reconstitution of succinate dehydrogenating system using succinate dehydrogenase and the components of the respiratory chain in different degrees of resolution [6—10], and use of artificial electron acceptor [11—13]. After extensive discussion [4,14,15], it is generally agreed that the reconstituted systems have most of the properties of the native respiratory chain. These systems are far too complicated for simple kinetic studies, being a qualitative rather than quantitative criterion of the enzyme activity.

Among the artificial electron acceptors phenazine methosulphate and ferricyanide are most widely used for the soluble succinate dehydrogenase [12,13, 16—18]. Another dye, WB, firstly introduced for the study of the respiratory chain by Jacobs [19] has also been reported to serve as effective oxidant for the enzyme [4,20,21]. No detailed information is available on the reaction mechanism of the succinate dehydrogenase reaction with WB as an oxidant.

As it has been pointed out by Dixon [25] the studies of interaction between an enzyme and artificial acceptor can provide new information on the properties of an enzyme. As an example, the function activity of HiPIP center [26] of the soluble succinate dehydrogenase has been directly demonstrated by using low concentrations of ferricyanide as electron acceptor [27—30]. Another property of the enzyme, i.e. change of its catalytic activity after the binding to the membrane [31] has been critically reevaluated using WB instead of phenazine methosulphate [32,33].

In this paper we wish to report the data on general properties of the succinate-WB reductase reaction catalyzed by soluble and membrane-bound succinate dehydrogenase. Preliminary reports on the subject have appeared elsewhere [32,33].

Materials and Methods

Succinate dehydrogenase was isolated from bovine heart muscle preparation [34] essentially according to King [21]. The enzyme was kept in small samples in liquid nitrogen. The activity was measured immediately after thawing using a reaction mixture containing 10 mM succinate, 10 mM Tris-sulfate buffer, pH 7.8, 0.1 mM EDTA (other additions are indicated in the legends to the figures) at 22°C. No activation of the enzyme was observed when the samples were preincubated with succinate prior to the assay. The preparations of the soluble enzyme were approx. 30% pure (flavin content) having an average of 8 atoms of iron per mol of covalently bound flavin and being reconstitutively active.

The ubiquinone-depleted Keilin-Hartree preparation was obtained by pentane extraction as described by Ernster et al. [35].

Free radical tetramethyl-*p*-phenylenediamine (Wurster's Blue) was prepared by oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride with bromine [36] and used after two recrystallisations from methanol. The small samples of WB-perchlorate were stored in argon atmosphere, protected from light for several months at room temperature. The compound is perfectly stable under such conditions, as revealed by its absorption spectra.

The kinetics of DCIP and ferricyanide reduction were measured with Hitachi 200-20 spectrophotometer in 1-cm light-path cuvettes at 600 and 420 nm, respectively. For WB reduction Pye Unicam SP 1700 spectrophotometer with 0.5-cm light-path cuvettes was used. The response of this instrument is perfectly linear within the range of 0—2 absorbance units. In all cases the reading began within 5 s after the reaction was started by the addition of the enzyme preparation.

Protein was determined by the biuret method [37]. All the chemicals used were the purest commercially available. Phenazine methosulfate and DCIP were kindly provided by Dr. T.P. Singer (Molecular Biology Division, Veterans Administration Hospital, San Francisco, U.S.A.).

Results

Properties of WB

The spectrum of the WB solution in the buffered system used for succinate dehydrogenase activity measurement is shown in Fig. 1. In agreement with others [38—40] the compound has two nearly symmetrical absorption peaks at 560 and 608 nm, with $\epsilon = 12\,000\ M^{-1} \cdot cm^{-1}$ and a shoulder at 520 nm. The reduced compound is colourless in the visible region of the spectrum. The pH change from 6.0 to 8.5 does not influence the spectral properties of the radical. The following compounds have no effect on its spectral properties when added to the solution of WB/KCN (1.5 mM), bovine serum albumin (0.5%), thenoyltrifluoroacetone (0.1 mM), succinate (10 mM), malonate (5 mM), phosphate (2 mM), $MgCl_2$ (1 mM), EDTA (0.3 mM). SH-containing compounds (cysteine, mercaptoethanol) and ascorbate quantitatively and instantly reduced WB. Storage of freshly prepared aqueous unbuffered solution of WB-perchlorate at room temperature during 7 h decreases the absorption at 608 nm not more than by 20%.

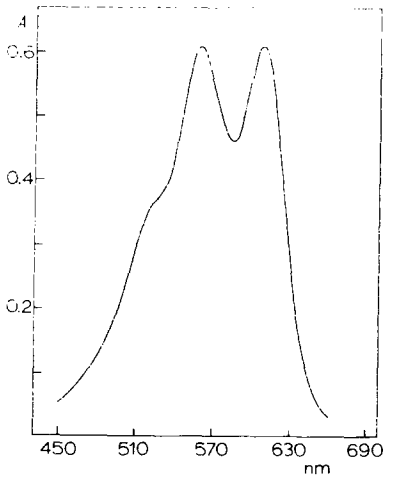

Fig. 1. Spectra of 50 μM WB-perchlorate in Tris-sulfate (10 mM)-buffered solution pH 7.8.

Comparison of reactivity of the soluble succinate dehydrogenase towards WB and other artificial electron acceptors

The reactivity of the soluble succinate dehydrogenase in the reconstitution test [6] and towards artificial electron acceptors [12,28] is extremely labile. In order to minimize the inactivation of enzyme in the course of purification, its reactivity with WB (30—100 μM), phenazine methosulfate (0.45—1.8 mM) and ferricyanide (25—100 μM) was compared using the preparation obtained after elution from calcium phosphate gel without further precipitation by $(NH_4)_2SO_4$. The activities estimated by extrapolation to infinite electron acceptor concentrations using double reciprocal plots $1/v$ versus 1/[acceptor] were: 7.7, 7.4 and 16.6 μmol succinate oxidized/min per mg protein for ferricyanide, phenazine methosulfate and WB, respectively. This finding is in perfect agreement with our previously reported data [33] and does not confirm the observation made by Ackrell et al. [41].

Kinetics of the overall reaction between succinate and WB catalyzed by the soluble enzyme

Since Slater [42] has introduced the method of K_s determination by the steady-state analysis of the reactions described by Eqn. 1, and Dixon [43] has considered the behaviour of the system where the reduced enzyme is oxidized by an electron acceptor after dissociation of the product, the studies of the K_m dependence on electron acceptor concentration are widely used for the establishing of the reaction pathway of dehydrogenase reactions.

Using this method, Zeijlemaker et al. [24] have shown that in the overall reaction between succinate and artificial oxidants catalyzed by soluble succinate dehydrogenase, the oxidation of the enzyme by ferricyanide or phenazine methosulfate occurs before fumarate leaves the active site of the enzyme. Gawron et al. [22] have found a positive curvature on the double reciprocal plots 1/[succinate] versus 1/[ferricyanide] when succinate is oxidized by ferricyanide in the presence of the soluble succinate dehydrogenase. This positive curvature has been interpreted by the authors as evidence for an

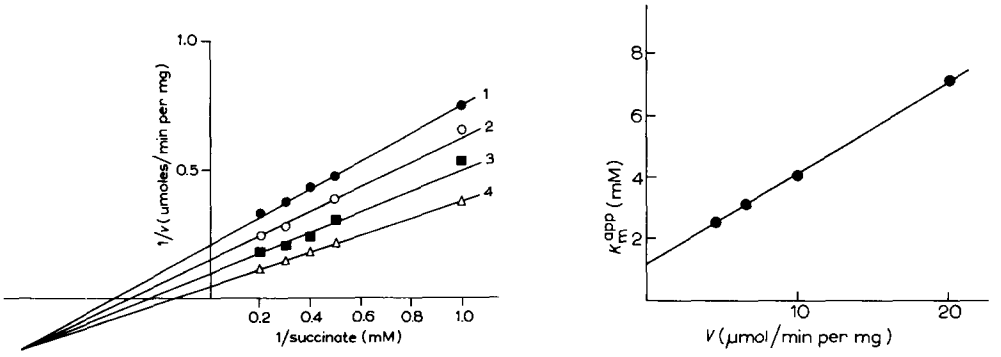

Fig. 2. Effect of varying succinate and WB concentrations on rate of oxidation of succinate by the soluble succinate dehydrogenase. 100 μM malonate was present. The concentrations of WB were (1) 0.05, (2) 0.066, (3) 0.1 and (4) 0.33 mM. 5.5 μg enzyme per ml assay mixture were added.

Fig. 3. Data of Fig. 2 plotted as K_m for succinate versus V at infinite succinate concentration at various WB concentrations.

alternative electron pathway via oxidation of fumarate-free reduced enzyme. Since the reaction mechanism may depend on the nature of electron acceptor and some ambiguity in electron pathways during succinate dehydrogenase reaction still exists, the kinetics of the overall succinate-WB reductase reaction catalyzed by the soluble enzyme were investigated. The constant concentration of the competitive inhibitor malonate was added in order to increase the apparent K_m value for succinate [44,45]. At infinite succinate concentration $1/v$ versus 1/[WB] plot gives a straight line intercepting abscissa at the K_m value for WB equal to 170 μM. As seen from Fig. 2, at all WB concentrations the plots $1/v$ versus 1/[succinate] also give straight lines converging in the third quadrant. This suggests the sequential mechanism of succinate-WB reductase reaction. The secondary plot K_m versus V (Fig. 3) also produces a straight line intercepting the ordinate at the point which corresponds to the K_s value equal to $1.2 \cdot 10^{-4}$ M (the K_i value for malonate under the same conditions was taken as $1.0 \cdot 10^{-5}$ M [46]). The value of K_s for succinate obtained as described above can be compared with that reported by Zeijlemaker et al. [24]: $7 \cdot 10^{-5}$ M (phenazine methosulfate as acceptor) and $6 \cdot 10^{-5}$ M (ferricyanide as acceptor) for slightly different conditions (0.1 M phosphate buffer, pH 7.8, 25°C), or determined using a more direct method: $3 \cdot 10^{-4}$ M [46]. Thus, the results obtained with WB as acceptor together with those obtained using other oxidants for the soluble [24] and membrane-bound enzyme [42,47] support the mechanism of the succinate dehydrogenase reaction proposed by Slater [42]. No evidence for an alternative pathway [22,23] could be obtained.

Kinetics of the soluble succinate dehydrogenase oxidation by WB as revealed by the steady-state analysis

The soluble reconstitutively active succinate dehydrogenase contains at least three redox components capable of participation in the catalytic cycle, i.e. histidyl-FAD [48,49], ferredoxin type iron-sulfur center [50,51] and super-oxidized iron-sulfur component of a HiPIP type [26] (center 3, according to Ohnishi et al. [52]). When an enzyme containing several redox components

interacts with an artificial oxidant, the non-specificity of the latter leads to ambiguity of the electron pathways: the acceptor may react with only one redox center or with two or more simultaneously. Clear enough the kinetic properties of an enzyme will be thus dependent on the particular electron pathways involved in reduction of the acceptor. Regarding the reactivity of the 'sites' towards artificial acceptor two of them can be clearly distinguished [27].

Thus the simplest hypothesis on the interaction between WB and the soluble succinate dehydrogenase in the presence of saturating concentrations of succinate can be described by Scheme 2:

$$\begin{array}{l} {\scriptstyle k_1 \downarrow e^-} \\ \mathrm{X} \xrightarrow{\mathrm{A_{ox}} k_2} \mathrm{A_{red}} \end{array}$$

$$\begin{array}{l} {\scriptstyle k_3 \downarrow\uparrow k_{-3}} \\ \mathrm{Y} \xrightarrow{\mathrm{A_{ox}} k_4} \mathrm{A_{red}} \end{array} \qquad (2)$$

where k_1 is a first-order rate constant for intramolecular reduction of a component X; k_3 and k_{-3} are rate constants for the forward and reverse redox reaction between two components of the enzyme X and Y; k_2 and k_4 are the rate constants for the simple second-order reactions of electron acceptor A with the components X and Y.

The system of equations describing the steady-state kinetics for Scheme 2 is not linear and its exact resolution is too cumbersome for direct analysis. However, it can be simplified on the following assumption: the redox potentials of the two components of the enzyme (X and Y) are close enough to consider the equilibrium constant for the reversible reaction between X and Y equal to 1. On this assumption the solution of the system is:

$$v = \frac{k_1 \cdot k_2 \cdot X_0 \cdot A}{k_1 + k_2 \cdot A + \dfrac{k_3 \cdot k_4 \cdot Y_0 \cdot A}{k_{-3} \cdot X_0 + k_4 \cdot A}} + \frac{k_1 \cdot k_3 \cdot k_4 \cdot X_0 \cdot Y_0 \cdot A}{\left(k_1 + k_2 \cdot A + \dfrac{k_3 \cdot k_4 \cdot Y_0 \cdot A}{k_{-3} \cdot X_0 + k_4 \cdot A}\right)(k_{-3} \cdot X_0 + k_4 \cdot A)} \qquad (3)$$

where X_0 and Y_0 are the total concentrations of the components X and Y, and A is the concentration of oxidized acceptor.

When the acceptor A reacts only with X($k_4 = 0$), the rate of reduction is:

$$v = \frac{k_1 \cdot X_0 \cdot A}{\dfrac{k_1}{k_2} + A} \qquad (4)$$

The Eqn. 4 is usual Michaelis type with V equal to $k_1 \cdot X_0$ and K_m equal to k_1/k_2. When the acceptor reacts only with Y($k_2 = 0$), the rate of reduction is:

$$v = \frac{k_1 \cdot X_0 \cdot A}{\dfrac{k_1 \cdot X_0}{k_4 \cdot Y_0} + \dfrac{k_1 \cdot A}{k_3 \cdot Y_0} + A} \qquad (5)$$

If $k_1 << k_3 \cdot Y_0$ (the reduction of the enzyme is slower than its oxidation, Eqn. 5 appears as:

$$v = \frac{k_1 \cdot X_0 \cdot A}{\frac{k_1 \cdot X_0}{k_4 \cdot Y_0} + A} \tag{6}$$

which is also usual Michaelis type with V equal to $k_1 \cdot X_0$ and K_m equal to $k_1 \cdot X_0/k_4 \cdot Y_0$.

Eqn. 3, which describes the rate of reduction of the acceptor A, which reacts simultaneously with X and Y, is non-linear with respect to A and the double reciprocal plot $1/v$ versus $1/A$ gives the curve intercepting the ordinate at $1/k_1 \cdot X_0$, i.e. at V equal to that of Eqn. 4 or 6. This analysis allows to discriminate three possible electron pathways for Scheme 2: (i) A reacts with center X; (ii) A reacts with center Y; (iii) A reacts with both X and Y at more or less equally significant rates. Considering the double reciprocal plots $1/v$ versus $1/A$, the mechanism (i) gives a straight line (K_m does not depend on concentrations of active species of X and Y; V is directly proportional to the concentration of X); the mechanism (ii) gives a straight line (K_m depends on the concentration of active species of X and Y; V is directly proportional to the concentration of X and does not depend on Y); the mechanism (iii) gives a curve with positive curvature.

As seen from Fig. 4, curve 1, the double reciprocal plot for the succinate-WB reductase reaction catalyzed by the soluble reconstitutively active enzyme gives a straight line. This corresponds to the mechanisms where the acceptor reacts with only one center of the enzyme. When the enzyme is stored under aerobic

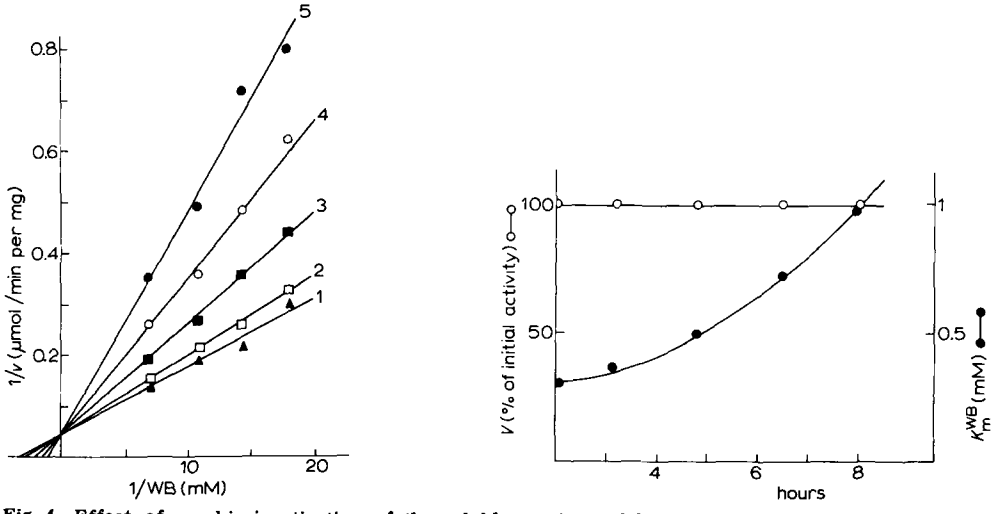

Fig. 4. Effect of aerobic inactivation of the soluble succinate dehydrogenase on rate of oxidation of succinate by WB. The enzyme (0.42 mg/ml) was stored aerobically in Tris-sulfate (10 mM, pH 7.5) at 0°C for: (1) 0, (2) 1.5, (3) 2.3, (4) 4.5 and (5) 6 h. 1 μg enzyme per ml assay mixture was added.

Fig. 5. Data of Fig. 4 plotted as V at infinite concentrations of WB (open circles) and K_m (closed circles) for the acceptor versus time of inactivation.

conditions, the K_m value for WB is markedly increased and V is constant during all time of inactivation. This is seen from Fig. 5, where the data of Fig. 4 are replotted as a function of V and K_m depending on the time of inactivation. The data presented in Fig. 5 are in agreement with the electron pathway (ii), where acceptor A reacts with the labile center Y.

Since the inactivation of the oxygen-sensitive site of succinate dehydrogenase (ESR-detectable center 3 [52], 'low K_m ferricyanide site' [27]) follows the first-order kinetics [28], it was of interest to compare the rate constant for inactivation of the component Y according to Model 2 with that obtained using low concentrations of ferricyanide. According to Eqn. 6, the K_m value for WB is $k_1 \cdot X_0/k_4 \cdot Y$. Inactivation of Y during aerobic storage can be described as follows:

$$Y_0^{\text{active}} \xrightarrow[\text{oxygen}]{k_i} Y_0^{\text{inactive}} \tag{7}$$

where k_i is first-order rate constant for inactivation process. The time dependence of the concentration of active species Y is:

$$Y_{0_t}^{\text{active}} = Y_{0_0} \cdot e^{-k_i \cdot t} \tag{8}$$

where Y_0 is the initial total concentration of component Y. The time dependence of K_m for WB can be described then as:

$$K_{m_t} = K_{m_0} \cdot e^{k_i \cdot t} \tag{9}$$

and

$$\ln K_{m_t}/K_{m_0} = k_i \cdot t \tag{10}$$

As seen from Fig. 6, the dependence of K_m value for WB on time indeed gives a straight line according to Eqn. 10 and the first-order rate constant k_i equal to $3.3 \cdot 10^{-3}$ min^{-1} is calculated. This value is in good agreement with the

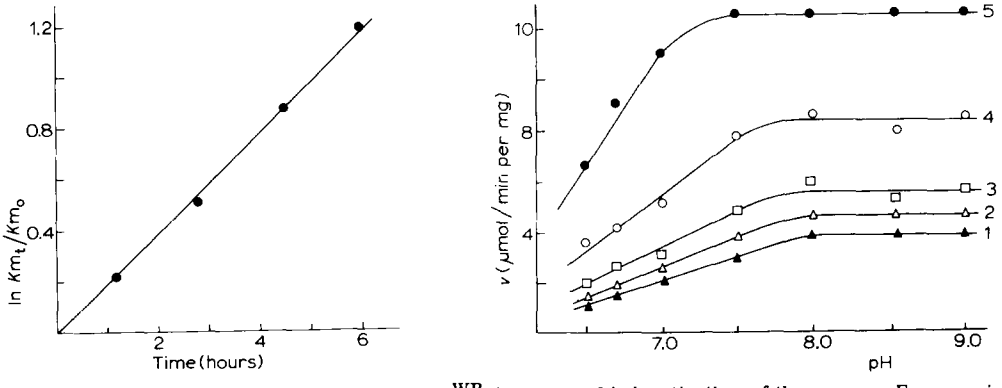

Fig. 6. The first-order rate constant plot for K_m^{WB} during aerobic inactivation of the enzyme. For experimental conditions see Fig. 4.

Fig. 7. Effect of varying pH on rate of succinate oxidation by the soluble enzyme in the presence of different WB concentration. The concentration of succinate was 20 mM. The concentrations of WB were (1) 50, (2) 66, (3) 100, (4) 200 μM; (5) infinite acceptor concentration. 0.8—2.0 μg enzyme per ml assay mixture were added.

value of first-order rate constant for inactivation of low K_m ferricyanide site [30]. Thus the process of reoxidation of the soluble reconstitutively active succinate dehydrogenase by WB can be qualitatively and quantitatively described in terms of Model 2, assuming that $k_2 = 0$ and Y is the low K_m ferricyanide reactive site.

The pH dependence of succinate-WB reductase reaction catalyzed by the soluble enzyme

As seen from Fig. 7, the rate of WB reduction is markedly increased within the pH range 6.5—7.8, being constant at more alkaline values of pH. The slopes of the curves depend on concentration of WB, being maximal for the values obtained by extrapolation to infinite acceptor concentrations ($k_1 \cdot X_0$) according to Eqn. 6. The pH dependence of V (the upper curve) is close to that theoretically calculated for the mechanism where only deprotonated form of the enzyme ($pK_a = 6.7$) is able to be reduced by substrate. This value can be compared with the pK_a value (about 7.0) of the active site of the sulfhydryl group as determined by *N*-ethylmaleimide inhibition of the soluble succinate dehydrogenase [46].

The reduction of WB by succinate-ubiquinone segment of the respiratory chain

In support the data reported by Mustafa et al. [53], we found that WB is rapidly reduced by succinate in the presence of submitochondrial particles treated by antimycin A, i.e. by the succinate-ubiquinone segment of the respiratory chain. Fig. 8 shows that succinate-WB reductase of the particles is sensitive to thenoyltrifluoroacetone indicating that most part of electron flow passes through ubiquinone. Since WB is an effective electron acceptor for succinate dehydrogenase (see above) and as seen from Fig. 8, this dye reacts with the respiratory chain at the site located between thenoyltrifluoroacetone and antimycin A-sensitive loci, Model 2 can be applied for the analysis of the reaction, assuming that the component X is succinate dehydrogenase and the component Y is ubiquinone. When concentration of WB is variable, the plot $1/v$ versus $1/A$ gives a straight line (Fig. 9, curve 1), indicating that only one kinetically

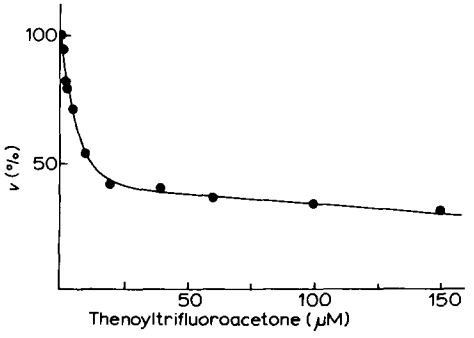

Fig. 8. Effect of thenoyltrifluoroacetone on the succinate-WB reductase reaction catalysed by Keilin-Hartree preparation in the presence of 10 mM succinate, 1 mM KCN, 2 nmol per mg of protein antimycin A and 100 μM WB. The amount of protein in the assay mixture was 0.03 mg/ml. Prior to the assay the particles were preincubated wih 10 mM succinate in the presence of antimycin A for 30 min at 20°C.

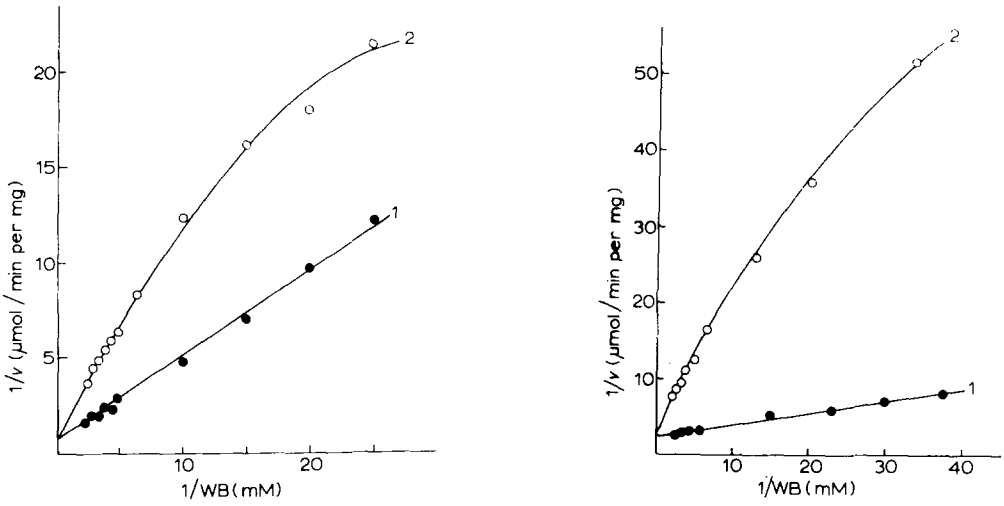

Fig. 9. Effect of thenoyltrifluoroacetone on the kinetics of WB reduction by the succinate-ubiquinone region of the respiratory chain. The experimental conditions as in Fig. 8 except various concentrations of WB and constant concentration of the inhibitor (100 μM, curve 2) were used. The amount of protein in the assay mixture was 0.03 mg/ml.

Fig. 10. Effect of ubiquinone extraction on the kinetics of WB reduction by succinate-ubiquinone region of the respiratory chain. The experimental conditions as in Fig. 8; 1, lyophilized control preparation; 2, pentane-extracted lyophilized preparation. The amount of protein in the assay mixture was 0.06 mg/ml.

significant site, presumably ubiquinone, is operating in the overall reductase reaction. In the presence of thenoyltrifluoroacetone (Fig. 9, curve 2), the plot gives a line with a positive curvature intercepting the ordinate at the same point as in the absence of the inhibitor. The same kinetic behaviour was obtained when succinate-WB reductase reaction of lyophilized preparation was compared with that of the ubiquinone-deficient particles (Fig. 10). The lyophilized control preparation gives a straight line (curve 1), whereas the pentane-extracted particles give a line with a positive curvature and intercepting the ordinate at the same point as in controls.

These results suggest that in terms of Model 2 in the native membrane-bound system WB reacts significantly only with the component Y (presumably ubiquinone). When most part of Q is extracted or the electron flow from succinate dehydrogenase to ubiquinone is largely inhibited, the rate of reduction of WB through the component Y becomes small and comparable with that through the component X (succinate dehydrogenase itself). As a result, both sites are operating and non-linear with respect to acceptor A, Eqn. 3 should be applied to describe the overall reaction. It should be emphasized that both after thenoyltrifluoroacetone inhibition or ubiquinone extraction the maximal turnover number of succinate dehydrogenase are equal to that for the native system. Thus, kinetic analysis gives no indication for positive (or negative) modulation of succinate dehydrogenase by ubiquinone.

Discussion

The major aim of this study is to analyze the interaction between the soluble and particulate succinate dehydrogenase and artificial electron acceptor WB, which seems to react differently from those routinely used for the measurement of the enzyme activity. When ferricyanide is used as acceptor in 1—5 mM range only 25—30% of the activity is recovered as compared to phenazine methosulfate provided that both dyes are extrapolated to infinite concentrations [12,13,54,55]. On the other hand, Zeijlemaker et al. [24] have reported that the catalytic center activities measured at infinite substrate and acceptor concentrations, are the same with these acceptors. The difficulties of meaningfull interpretation of the V method for determination of the activity of the soluble enzyme have been briefly discussed [56,57]. The problem has been revived after it has been shown that ferricyanide interacts with at least two centers of the soluble succinate dehydrogenase and with only one of those in the native particulate and reconstituted systems [27,30]. The evidence have been presented that 'low K_m ferricyanide' reductase activity of the reconstitutively active enzyme is a simple measure of functional activity of HiPIP ESR-detectable component of the protein [27,29]. Thus, it has become evident that some properties of the soluble succinate dehydrogenase are related to the nature of the artificial acceptor used. Among the latters WB bring our attention, because the reactions between the respiratory chain-linked dehydrogenases and the stable free radical species appear to be of a general interest. As shown in this paper this dye seems to meet all the criteria for a simple and convenient assay of succinate dehydrogenase reaction.

Perhaps the most important property of WB as acceptor is that compared with others it reveals the highest catalytic activity of the soluble succinate dehydrogenase. This observation has been firstly reported when the catalytic activities of the soluble and membrane-bound succinate dehydrogenase were compared using WB and phenazine methosulfate as acceptors [32]. Before our paper [32] came out of press, Ackrell et al. [41] criticised our observations claiming they were unable to reproduce our results. Although it is not an immediate purpose of this paper to answer the question of why the activity of the enzyme with BW is higher than with phenazine methosulfate or ferricyanide, some points should be emphasized.

The activity measured with ferricyanide can be considered only as an approximation since the destructive effect of this oxidant on the catalytic activity of the enzyme has already been demonstrated [27]. It must be pointed out that it is not known, whether an enzyme-dye complex of the Michaelis type is formed when phenazine methosulfate is used as acceptor. Theoretically if no such complexes exist, any acceptor at infinite concentrations would be expected to reveal the same activity of the enzyme ($V = k_1 \cdot X_0$ according to Scheme 2). If, however, an intermediate complex between the enzyme and acceptor is reversibly formed, the value of V must be lower than $k_1 \cdot X_0$. A more detailed analysis of the kinetics for possible reaction mechanisms of an enzyme with several active redox sites interacting with artificial acceptor will be published elsewhere.

The criticism of our interpretation [32,33] of the 'effect of environments on

succinate dehydrogenase activity' [31] has been substantiated by ESR experiments on reoxidation of Fe-S centers by phenazine methosulfate [58].

It is difficult to visualize how the results of the single turnover experiment can be directly related to the turnover number of the enzyme in the steady-state conditions, without clear knowledge on the kinetic mechanism of the enzyme-acceptor interaction. Indeed, if the reoxidation of the enzyme by phenazine methosulfate is no 'rate-limiting step', then it is not clear why the steady-state rate of reduction of this dye rather strongly depends on its concentration. In fact, the validity of EPR kinetic experiments of Ackrell et al. [58] for evaluation of the turnover number of the enzyme have been seriously questioned by the results of these authors on reoxidation of HiPIP-type Fe-S center of Complex II. They have demonstrated [58] that ferricyanide, which is certainly a poor oxidant for the membrane-bound succinate dehydrogenase under steady-state conditions, oxidizes the enzyme faster than expected from the steady-state measurements of the turnover number with phenazine methosulfate, which is a far better electron acceptor for this preparation.

The data on the kinetics of the overall succinate dehydrogenase reaction support the mechanism proposed by Slater [42] and confirmed by Zeijlemaker et al. [24], where the enzyme is oxidized in a form of the reduced enzyme-product complex. Of relevance to the present discussion are the observations by Gawron et al. [22] who have demonstrated the different kinetic behaviour of the soluble and particulate preparations of succinate dehydrogenase. These investigators have observed the positive initial slope in the plots $1/v$ versus 1/[acceptor] for soluble enzyme using ferricyanide as oxidant. The hypothesis has been suggested that kinetic mechanisms of the overall succinate dehydrogenase reaction are different for the soluble and particulate enzyme and that, in the latter, oxidation of the enzyme occurs after dissociation of fumarate [22]. We are inclined to believe that the deviation from the linearity in the $1/v$ versus 1/[acceptor] plot observed by Gawron et al. [22] is a result of two sites of electron acception rather than a contribution of two different kinetic mechanisms in the overall reaction. This explanation would fit Model 2 under the condition where the resolution for the steady-state rate is described by Eqn. 3. Thus, there is no evidence at present for the mechanism of succinate dehydrogenase reaction different from that proposed by Slater [42].

In terms of Model 2 the kinetics of WB reduction by the reduced succinate dehydrogenase suggest that this acceptor interacts with the same center of the enzyme as does ferricyanide in low concentrations range. The substantial body of evidence exists that the latter accepts electrons from the HiPIP component of the enzyme. Thus, it appears that the electron transfer pathway from the soluble succinate dehydrogenase to WB is similar to that in the native succinate-ubiquinone region of the respiratory chain. The question then arises what is the chemical nature of the specificity of WB. There is very little detail known at the moment of the immediate environment of the HiPIP center of succinate dehydrogenase. Taking into consideration the structure of non-heme iron proteins [60—62] it would be reasonable to expect a higher rate of iron-sulfur oxidation by the cation of WB than by trianion of ferricyanide due to electrostatic interaction between Fe-S center and those two acceptors.

When WB serves as an electron acceptor for succinate-ubiquinone region of

the respiratory chain, the kinetics of the overall reaction are typical for the 'one site' model. As may be evidenced by a marked sensitivity of this reaction to thenoyltrifluoroacetone and pentane extraction (at any fixed concentration of the acceptor), the site of interaction of WB with this system may be identified as ubiquinone. Since WB is an efficient electron acceptor for the soluble succinate dehydrogenase, this would suggest that either the HiPIP center of the enzyme in the native system is not available for WB, or ubiquinone serves as an oxidant of the enzyme in a much more effective way than does artificial dye. The recent observations of Yu et al. [10] on reconstituted succinate-ubiquinone reductase also fit this explanation. The kinetic behaviour of thenoyltrifluoroacetone-inhibited or ubiquinone-deficient particulate succinate dehydrogenase is typical for a 'two site' model. This indicates that the slow oxidation of the membrane-bound enzyme does occur at the site located before ubiquinone. This site may be identical to that reacting with high concentrations of ferricyanide [27], which is not penetrating through the intramitochondrial membrane [63]. On the other hand, in contrast to phenazine methosulfate [64] infinite concentrations of WB give the same V value for succinate dehydrogenase independently of the natural content of ubiquinone or its functional link with the enzyme. In terms of Model 2, these data suggest that no specific modulation of the succinate dehydrogenase activity by ubiquinone exists in the intramitochondrial membrane.

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